Intestinal Human Colon Adenocarcinoma Cell Line LS180 Is an Excellent Model to Study Pregnan X Receptor, but Not Constitutive Androstane Receptor, Mediated CYP3A4 and Multidrug Resistance Transporter 1 Induction: Studies with Anti-Human Immunodeficiency Virus Protease Inhibitors

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ABSTRACT:

Lack of an established cell line model to study induction of cytochromes P450 (P450s) and drug transporters poses a challenge in predicting in vivo drug-drug interactions. Although not well characterized, LS180 cells could be an excellent cell line to study induction of P450s and transporters because they express pregnane X receptor (PXR). Therefore, as part of a larger study of in vitro to in vivo prediction of inductive drug interactions, we determined induction of various P450s and drug transporters by the anti-human deficiency virus protease inhibitors (PIs) and the prototypic inducer, rifampin, in LS180 cells. Among these proteins, the various PIs significantly induced (n = 3–5) only CYP3A4 and multidrug resistance transporter 1 (MDR1) transcripts (2- to 50-fold). CYP3A4 activity (1-hydroxymidazolam formation) was increased (2-fold) by rifampin (10 µM) but was reduced by the PIs (1.5- to 7-fold). Surprisingly, constitutive androstane receptor 1 (CAR1) was not found to be expressed in these cells. Additionally, using a reporter assay, we found that PIs did not activate CAR3 (the natural splice variant of CAR1) but significantly activated PXR (2- to 24-fold), which correlated well with induction of CYP3A4 and MDR1 transcripts (r = 0.9). Furthermore, in a PXR-knockdown stable LS180 cell line, induction of CYP3A4 and MDR1 mRNA after treatment with PIs and rifampin was significantly reduced (1.4- to 5-fold) compared with that in PXR nonsilenced cells. Based on these data, we conclude that LS180 cells could be used as a readily available, high-throughput cell line to screen for PXR-mediated induction of CYP3A4 and MDR1 transcripts. These data also indicate that the majority of the PIs are likely to produce intestinal drug-drug interactions by inactivating or inhibiting CYP3A enzymes even though they induce CYP3A4 and MDR1 transcripts via PXR.

To date, much of the focus on clinically significant drug interactions has been on metabolic-based inhibitory drug interactions. However, there is increasing appreciation that inductive drug interactions are more common than previously thought, and such interactions can also extend to transporters. For this reason, the U.S. Food and Drug Administration has recently issued draft guidance on models to study inductive drug interactions. This draft guidance states that “At this time, the most reliable method to study a drug’s induction potential is to quantify the enzyme activity of primary hepatocyte cultures following treatments including the potential inducer drug, a positive control inducer drug [rifampin for CYP3A4], and vehicle-treated hepatocytes (negative control), respectively” (http://www.fda.gov/
Materials and Methods

**Chemicals.** Protease inhibitors (ritonavir, nelfinavir, amrenavir, saquinavir, indinavir, lopinavir, tipranavir, and atazanavir) were obtained from the National Institutes of Health AIDS reagent program. Rifaxin was purchased from Bedford Laboratories (Bedford OH). Midazolam and 1'-OH-midazolam were purchased from Cerilliant (Round Rock, TX). CITC was purchased from BIOMOL International LP (Plymouth Meeting, PA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific and were of highest purity available.

**Cell Culture and Molecular Biology Reagents.** LS180 cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA). All of the cell culture reagents, as recommended for growth and maintenance of LS180 cells (e.g., culture medium and trypsin) were purchased from ATCC. Fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT). Lipo-pectamine and Plus reagent and transfection medium (Opti-MEM) were obtained from Invitrogen (Carlsbad, CA). PCR Master Mix, reverse transcription reagents, and TaqMan probes were purchased from Applied Biosystems (Foster City, CA). Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). DNA and RNA extraction kits were purchased from Qiagen (Valencia, CA). The Luciferase Reporter Assay System and the β-galactosidase assay kit were purchased from Promega (Madison, WI). Other cell culture reagents were purchased from Invitrogen unless otherwise noted.

**Plasmid Constructs.** The expression plasmid for human PXR (pSG5-hPXRΔATG) and the reporter plasmid (XREM-CYP3A4)-tk-luc reporter were kindly provided by Dr. Bryan Goodwin (GlaxoSmithKline, Research Triangle Park, NC). Details of the plasmid have been published previously (Goodwin et al., 1999). An expression vector containing β-galactosidase cDNA under T7 promoter, pCH110, was purchased from GE Healthcare (Piscataway, NJ). The expression plasmids for human CAR1, CAR3, and RRX and their respective controls were generously provided by Dr. Curtis Omiecinski (Pennsylvania State University, University Park, PA) (Auerbach et al., 2005). The CYP2B6-PBREM/XREM reporter plasmid was a kind gift from Dr. Hongbing Wang (University of Maryland, Baltimore, MD) (Wang et al., 2003).

**Cell Culture.** LS180 cells were grown as suggested by ATCC except that they were subcultured using 5 mM EDTA to minimize clumping. Briefly, the cells were maintained in Dulbecco’s modified Eagle medium with 10% FBS, 2 mM L-glutamine, 50 units/ml penicillin G, and 50 μg/ml streptomycin.

Creating PXR Knockdown Cell Line in LS180 Cells. The PXR knockdown cell line was created by transfecting lentivirus containing shRNA for PXR. The primer pair designed for PXR shRNA was as follows: 5'-CCG GCA TGA AGG AGA GTA TCG CTA GAT CTT CTC ATC GCC GTT TGT G-3' and 5'-AAT TCA AAA ACG GCA TGA AGG AGA GTA TCT CGA CAT CTT CTC ATC GCC G-3'.

In brief, lentivirus was produced by cotransfection of lentiviral plasmid (pKLO.1) containing shRNA for PXR along with packaging plasmid (ps-PAX2) and envelope plasmid (pMD2.G) in the ratio of 4:3:1, respectively, in HEK293T cells using the calcium phosphate transfection method. Twelve to 15 h after transfection virus was collected, filtered, and stored at −80°C until used. This virus filtrate was then added to adherent LS180 cells in the presence of 8 μg/ml Polybrene (Sigma-Aldrich). A pool of infected cells was selected in the presence of puromycin (1 μg/ml). Endogenous PXR mRNA levels were confirmed by quantitative real-time PCR using TaqMan probes from Applied Biosystems. This cell line was propagated in the presence of puromycin and called PXRsi. Similarly, the negative control cell line, called GFPsi, was created by using shRNA for GFP. Because of the lack of GFP in LS180 cells, shRNA for GFP should not affect the expression levels of any specific target gene in LS180 cells and would be a control for off-target silencing.

**Drug Treatment of LS180 or PXRsi or GFPsi Cells for mRNA Analysis.** Cells (0.5 million) were seeded in six-well plates and allowed to attach at 37°C in a humidified atmosphere of 5% CO2. Except for nelfinavir, stock solutions of the PIs or rifampin (500× prepared in DMSO) were diluted in medium before use. Because of limited solubility, nelfinavir stock solution (500× prepared in DMSO) was mixed with β-cyclodextrin (0.05%) before dilution in the medium. Therefore, β-cyclodextrin (0.05%) was added to the medium containing the other drugs as well. Twenty-four hours after cell seeding, media containing various drugs were added to the respective treatment wells at a final DMSO concentration of 0.2%. DMSO at this concentration had no effect on expression levels of various enzymes and was not cytotoxic. The media were not renewed for the duration of the experiment. Cells were harvested at different time points (24, 48, 72, or 96 h), as specified, after treatment, to determine the time point for maximal induction. To determine the level of induction of the target genes by the various PIs, cells were treated with the drug (10 μM) for 96 h followed by RNA extraction. Detailed concentration-response studies were performed for ritonavir, nelfinavir, or rifampin to obtain the EC50 and Emax of induction of CYP3A and MDR1 transcripts. Cells were treated with various concentrations of the drug (0–75 μM) for 96 h and harvested in RNA extraction lysis buffer (provided in an RNasey Mini Kit). RNA was extracted from the harvested cells using an RNasey Mini Kit according to the manufacturer’s protocol and stored at −80°C for RNA analysis by real-time assay. The concentration of purified RNA was determined by a spectrophotometer (SmartSpec Plus Spectrophotometer; Bio-Rad, Hercules, CA), as was the purity, using a 260/280 absorbance ratio (ratio of 1.8 to 2.0).

**Quantification of mRNA Induction.** Of the total RNA, 2 μg (in a 50-μl reaction) was reverse-transcribed into cDNA using Applied Biosystems TaqMan reverse transcription reagents according to the manufacturer’s instructions. The resulting cDNA was used for real-time qPCR analysis for all of the target genes.

qPCR assays for various P450 (CYP1A1, 1A2, 2B6, 3A4, 3A5, 2C9, 2C19, and 2D6) enzymes and transporters (MDR1, MRP2, BCRP, and OATP1A2) were performed using gene-specific primers and FAM-labeled fluorescent MGB probes in an ABI 7000 Sequence Detection System (Applied Biosystems). β-Glucuronidase was used as the endogenous control. The real-time reaction contained 10 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 40 ng of RNA equivalent cDNA, and primers (200 nM) and probes (100 nM) in a final volume of 20 μl. The PCR reaction was as follows: 95°C hot start for 10 min, followed by 40 cycles at 95°C for 15 s and then 60°C for 60 s. Each sample was analyzed in triplicate. The mRNA levels of each test gene were normalized to β-glucuronidase, according to the following formula: Ct (test gene) = Ct (β-glucuronidase) − ΔCt. Therefore, the relative mRNA induction levels of each gene were calculated using the ΔΔCt method: ΔCt (test gene) − ΔCt (test gene in the DMSO control) =ΔCt (test gene in the DMSO control) − ΔCt (test gene in the control treatment)
\[ \Delta C_T \] (test gene). The fold changes of mRNA levels were expressed as the relative expression \( 2^{-\Delta C_T} \).

CYP 3A Activity Assay. CYP3A activity was measured by estimating 1'-OH-midazolam (MDZ) formation in drug-treated intact cells. Cells (0.5 million) were seeded in a six-well plate in Dulbecco's modified Eagle's medium containing 10% FBS. After 24 h, cells were treated with DMSO (vehicle) or various drugs as described above. After a 96-h drug treatment, cells were washed twice with phosphate-buffered saline and then incubated in Dulbecco's modified Eagle's medium (without FBS) at 37°C for 4 h (time optimized in a time course study). After washing, the cells were incubated with media containing 8 \( \mu \)M MDZ. The media were sampled at 1 h and stored at 4°C pending analysis for 1'-OH-MDZ. In any given experiment, each reaction was performed in duplicate. A calibration curve ranging from 2.5 to 250 ng/ml of 1'-OH-MDZ was prepared in blank cell media. The samples were subjected to liquid-liquid extraction and the amount of 1'-OH-MDZ was measured by liquid chromatography-mass spectrometry as described previously (Kirby et al., 2006b) with the following minor modifications. To a 0.5-ml aliquot of cell lysate, a stable labeled internal standard (d5-1'-OH-midazolam, m/z 346.9, 25 ng) was added. Samples were acidified with 100 \( \mu \)l of ammonium hydroxide and vortexed. Then, 5 ml of n-tert-butyl ether were used for extraction.

PXR, CAR1, or CAR3 Activation Assays. For the PXR activation assay, LS180 cells were seeded into 24-well plates at a density of 2 \( \times \) 10^5 cells/well. After 24 h of plating, overnight transfection was performed using Lipofectamine Plus reagent, as per the manufacturer's protocol. Briefly, the transfection mixtures contained 100 ng of human PXR expression vector (pSG5-humanPXR), 400 ng of luciferase reporter gene construct (XREM-CYP3A4-tk-luc), and 400 ng of pCH110 (an expression vector containing \( \beta \)-galactosidase cDNA under T7 promoter). After transfection, plasmid-containing media were replaced with drug- or DMSO-containing media (replaced after 24 h) and incubated for 48 h. The final concentration of DMSO (0.2% v/v) was kept constant in each sample. In every experiment, rifampin (10 \( \mu \)M) was included as a positive control. At the end of 48 h, the cell layers were washed twice with ice-cold phosphate buffer saline (pH 7.4), scraped, and collected in 200 \( \mu \)l of reporter lysis buffer provided with the \( \beta \)-galactosidase assay system (Promega). Ten microliters of the cell lysate was used to determine the luciferase activity with a Luciferase Assay System (Promega). An aliquot of cell lysate (50 \( \mu \)l) was used to determine the \( \beta \)-galactosidase activity according to the manufacturer’s recommendation. Luciferase activity was normalized to the \( \beta \)-galactosidase activity and expressed as fold activation with respect to the solvent (0.2% DMSO)-treated controls. The potency (EC_{50}) and maximal activation of PXR (E_{max}) by rifonavir, nelfinavir, or rifapmin were determined by treating the transfected cells with various concentrations (0.1-50 \( \mu \)M) of the drug. PXR activation by the remaining PIs was studied at only 10 \( \mu \)M.

For CAR1 and CAR3 activation assays, the procedure was similar to the PXR activation assay with the following modifications. For CAR1 or CAR3 activation, 100 ng of human CAR1 (pCMV2-CAR1) or human CAR3 (pCMV-CAR3) expression plasmid, respectively, was added to the transfection mix containing 200 ng of luciferase reporter plasmid (CYP2B6-PBREM/XREM) and 200 ng of pCH110 (\( \beta \)-galactosidase expression plasmid). To test for RXR-dependent activation, 100 ng of human RXR expression plasmid (pDNA3.1-RXR) was added to the transfection mix. Negative controls for each experiment involved using the empty plasmid in the transfection mix. After overnight transfection with plasmids (expressing CAR1 or CAR3 along with RXR, 2B6 response elements, and \( \beta \)-galactosidase) using Lipofectamine 2000 (Invitrogen), the cells were treated with the drug-containing medium for 24 h followed by determination of the luciferase activity and \( \beta \)-galactosidase activity as explained above. The data for each set of transfections were normalized to the respective DMSO-treated controls.

Cell Cytotoxicity (MTT) assay. Cell viability was quantified by the colorimetric MTT assay. Cytotoxicity of drugs in LS180 cells was tested in a collagen-coated 96-well plate. Cells (5 \( \times \) 10^5/well) were seeded on MTS plates overnight followed by addition of rifapmin (0–100 \( \mu \)M) or various PIs (0–50 \( \mu \)M) for 96 h as described above (see Drug Treatment of LS180 or PXRsi or GFPsi Cells for mRNA Analysis). The MTT assay was performed at the end of 96 h as per the manufacturer’s instructions. Cell viability was also tested in LS180 cells after transfection and drug treatment. Collagen-coated 24-well plates (2 \( \times \) 10^5 cells/well) were used for toxicity determination. After transfection (as described for the PXR activation assay), cells were treated with various drugs (as described above) for 24 or 48 h. At the end of the treatment, the MTT assay was performed as per the manufacturer’s instructions. Values are expressed as a percentage of the control.

Statistical and Data Analysis. Results from real-time assays, reporter gene assays, and the CYP3A activity assay are expressed relative to those observed in the vehicle control. Data are presented as mean ± S.D. of three to five different experiments (triplicate determinations in each experiment unless otherwise indicated). Where appropriate, statistical comparisons were made on log-transformed data (Figs. 1, 2, 4, 5, and 6) using analysis of variance followed by the Bonferroni correction. The significance of size was set at \( p < 0.05 \) (as indicated). For concentration-dependent mRNA induction experiments (Fig. 3), EC_{50} and EC_{max} were determined using WinNonlin (Pharosight Corporation, Mountain View, CA).

To gain insight into whether rifampin, ritonavir, or nelfinavir share a common mechanism for induction of CYP3A4 or MDR1 transcripts, we examined whether the induction of each of these transcripts was correlated among the three drugs. In addition, to explore whether each drug induced CYP3A4 and MDR1 transcripts by a common mechanism, we determined the correlation in induction of the two transcripts by each of the three drugs. To further confirm that rifampin and PIs induce MDR1 and CYP3A4 via PXR activation, we determined the correlation between PXR activity and CYP3A4 or MDR1 induction across all the drugs. These correlations were estimated using Microsoft Office Excel 2007 (Microsoft, Redmond, WA).

Results

Time Course of Induction of CYP3A4 and MDR1 Transcripts by Ritonavir, Nelfinavir, or Rifapmin. At 10 \( \mu \)M, the three drugs induced CYP3A4 mRNA to different extents (3- to 16-fold). This induction reached a plateau between 24 and 72 h and then increased (10- to 30-fold) at 96 h (statistically significant for rifampin but not for ritonavir or nelfinavir) (Fig. 1). In contrast, MDR1 mRNA was maximally induced (4.5- to 14-fold) by all the three drugs at 24 h, and this induction remained stable until 96 h. Because the 96-h time point showed the highest level of induction of CYP3A4 mRNA, all further induction studies were performed over this period. We did not investigate longer time points because of the concern about viability of the cells beyond 96 h.

Induction of CYP3A4 and MDR1 Transcripts by the PIs or Rifapmin. Treatment of LS180 cells for 96 h with 10 \( \mu \)M concentrations of various PIs (RTV, NFV, APV, IDV, SQV, LPV, TPV, and ATV) or RIF significantly (\( n = 3–5 \)) induced CYP3A4 (2- to 28-fold) in the order, RIF > TPV > APV > RTV > LPV > NFV > SQV > IDV > ATV and MDR1 (4- to 20-fold) in the order RIF > APV > RTV > LPV > NFV > ATV > SQV (Fig. 2A). Indinavir did not significantly induce MDR1 transcripts. When these data were expressed with respect to induction by 10 \( \mu \)M rifampin, tipranavir and amprenavir were as potent as rifampin (\( p > 0.05 \)) in inducing CYP3A4 transcripts followed by RTV > LPV > NFV > SQV > IDV > ATV. For induction of MDR1 transcripts, ritonavir and amprenavir were as potent as rifampin followed by TPV > NFV > SQV > IDV > ATV > SQV (Fig. 2B). At 10 \( \mu \)M, the transcripts of none of the other P450s and transporters tested were significantly induced by the PIs. This observation occurred despite the fact their transcript expression was robust and easily detected (threshold cycle, \( C_T \) ranging from 29–35) as estimated in cDNA synthesized from 40 ng of mRNA in real-time qPCR) and followed the order 2B6 > 2D6 > 2C9 > 1A1 = 1A2 > 3A5 > 3A4 = 2C19 or MDR1 > MRPs > BCRP. However, the expression of CYP2C8 and OATP1A2 transcripts was low or undetectable. At the concentrations used, the cytotoxicity (determined by the MTT assay) of the drugs was minimal with estimated cell survival of 85 to 100%.

Concentration-Dependent Induction of CYP3A4 and MDR1 by Rifapmin, Ritonavir, or Nelfinavir in LS180 Cells. To allow future
FIG. 1. Induction of CYP3A4 and MDR1 transcripts by 10 µM RIF, RTV, or NFV was significant at 24, 48, 72, and 96 h. For RIF, induction of CYP3A4 transcripts was constant over 24 to 72 h and then increased at 96 h (*p < 0.05), whereas those of RTV and NFV showed a similar pattern but induction levels at 96 h were not significantly different from that at 24 to 72 h. MDR1 transcripts remained unchanged from 24 to 96 h. Data are presented as mean ± S.D. (n = 4).

FIG. 2. When expressed relative to DMSO (A) or 10 µM rifampin (B), PIs (10 µM) significantly induced CYP3A4 transcripts with RIF, TPV, APV, RTV, and LPV producing >10-fold induction, NFV and SQV producing >5-fold induction, and IDV and ATV producing <5-fold induction. The induction of MDR1 transcripts was significant (*p < 0.05) for rifampin and the PIs (except IDV) with RIF, APV, RTV, LPV, and TPV producing >10-fold induction, NFV and ATV producing >5-fold induction, and SQV producing <5-fold induction. Data are presented as mean ± S.D. (n = 3–5). LS180 cells were treated with various PIs (10 µM) for 96 h, and mRNA levels were measured as described under Materials and Methods.
in vitro to in vivo prediction of P450- and transporter-based inductive
drug interactions by ritonavir and nelfinavir, we conducted a concentra-
tion-dependent study to estimate the \( E_{\text{max}} \) and \( EC_{50} \) of induction of CYP3A4 and MDR1 transcripts by these PIs and rifampin. Transcript levels of CYP3A4 and MDR1 were determined in cells treated with varying concentrations of ritonavir (0–25 µM), nelfinavir (0–15 µM), or rifampin (0–50 µM). The concentration ranges for the three drugs were based on obtaining greater than 85% cell survival (estimated by MTT assay) in the presence of the drugs. All three drugs induced CYP3A4 and MDR1 transcripts in a concentration-dependent manner (Fig. 3). \( E_{\text{max}} \) and \( EC_{50} \) of CYP3A4 transcript induction by nelfinavir could not be estimated because of cytotoxicity observed at the higher concentrations (>15 µM) of the drug. The \( EC_{50} \) values of induction of CYP3A4 (~7 µM) or MDR1 (~1.5 µM) by ritonavir and rifampin were remarkably similar, with MDR1 transcripts being more inducible at a lower concentration of the drugs than CYP3A4 transcripts. The maximal induction of transcripts, as determined by \( E_{\text{max}} \), differed between the drugs and increased in the order RIF > RTV for CYP3A4 and RIF > RTV > NFV for MDR1. Maximum induction of CYP3A4 transcripts was higher than for MDR1 transcripts by rifampin (49- and 24-fold, respectively) at 50 µM or by nelfinavir (15- and 6-fold, respectively) at 15 µM concentration. However, maximal induction of CYP3A4 and MDR1 transcripts by ritonavir (25 µM) was comparable (~13 fold).

**CYP3A Activity in LS180 Cells in the Presence of Various PIs or Rifampin.** To test whether the above induction in CYP3A4 transcripts translated into activity, we optimized a CYP3A activity assay in LS180 cells using midazolam as a substrate. Because the inducers could act as CYP3A inhibitors, after induction and before measuring CYP3A activity, we incubated the cells with drug-free media for 4 h to promote drug efflux (a time determined to be optimal in a time course experiment ranging from 0 to 12 h). Ritonavir induced CYP3A activity by 2-fold (compared with the DMSO control) at 10 µM but not at other concentrations tested (1 and 5 µM) (Fig. 4). At the concentrations tested (1, 5, and 10 µM), ritonavir decreased CYP3A activity by 7-fold, whereas nelfinavir decreased CYP3A activity by approximately 4-fold. At 10 µM, saquinavir decreased CYP3A activity by 3-fold, whereas lopinavir, tipranavir, and atazanavir decreased this activity by only 1.5-fold. At 10 µM, amprenavir and indinavir had no effect on CYP3A activity.

**Role of PXR in the Induction of CYP3A4 and MDR1 Transcripts in LS180 Cells.** To gain insight into the mechanisms involved in induction of CYP3A4 and MDR1 transcripts by the PIs, we measured the activation of PXR as well as the induction of CYP3A4 and MDR1 transcripts in PXR-competent and PXR-knockdown LS180 cells by the PIs or rifampin.

**PXR activation by PIs or rifampin in a reporter gene assay in LS180 cells.** At 10 µM, the order of PXR activation was as follows: RIF (20-fold) > RTV (11-fold) > APV (12-fold) > LPV (5-fold) > TPV (5-fold) > SQV (4-fold) > ATV (4-fold) > IDV (2.4-fold) (Fig. 5). Statistically significant activation was observed with all of the PIs except 10 µM nelfinavir because nelfinavir was significantly more toxic to the transfected cells (~10% cell survival) than the other PIs for which the cytotoxicity was minimal (>90% cell survival observed). When nelfinavir was studied in a concentration-response study, significant PXR activation (2.7- to 11.7-fold) was observed at lower concentrations (0.1–5 µM). This result demonstrates that nelfinavir was more toxic in this assay than in the induction assay possibly because of treatment of the cells with Lipofectamine combined with 48 h of drug exposure. Concentration-response studies for PXR activation were performed by treating transfected cells with various concentrations of ritonavir (0.1–50 µM), nelfinavir (0.1–5 µM), or rifampin (0.1–50 µM). All three drugs activated PXR in a concentration-dependent manner (data not shown). The \( EC_{50} \) values of PXR activation (~0.4 µM) by ritonavir and nelfinavir were remarkably similar but lower than those for rifampin (~0.9 µM). The \( EC_{50} \) of PXR activation by rifampin was comparable with that reported previously (0.72 µM) (Lemaire et al., 2006). The maximal PXR activa-
tion, as determined by $E_{\text{max}}$, was similar between ritonavir and nelfinavir (~13-fold) and slightly higher for rifampin (~16-fold).

Induction of CYP3A4 and MDR1 transcripts by various protease inhibitors and rifampin in PXR-knockdown LS180 cells. To confirm the predominant involvement of PXR in PI-mediated induction of CYP3A4 and MDR1 transcripts in LS180 cells, we established a PXR-knockdown LS180 cell line (PXRsi) using RNA interference. When compared with the negative vector-transfected control cell line, GFPsi, we consistently achieved greater than 80% knockdown of PXR expression in the PXRsi cell line. The basal levels of CYP3A4 and MDR1 decreased (1.5- to 3-fold; $p > 0.05$) in PXRsi cells compared with the control cells. When these cells were treated with various PIs or rifampin (10 $\mu$M) for 96 h, induction of CYP3A4 and MDR1 transcripts by rifampin or the more potent PI inducers (RTV, NFV, APV, LPV, and TPV; induction levels greater than 5-fold) was reduced by 60 to 80% in PXRsi cells compared with the control cells (Fig. 6). As expected, weak inducers (IDV, ATV, and SQV) did not affect the induction of CYP3A4 and MDR1 transcripts in PXRsi cells compared with control cells (data not shown).

Role of CAR in the Induction of CYP3A4 and MDR1 Transcripts by the PIs in LS180 Cells. Although PXR appeared to be the major player in the induction of CYP3A4 and MDR1 transcripts in LS180 cells, we also investigated the role of CAR1 in the induction of these transcripts because it has been shown previously to be involved in the transcriptional regulation of CYP3A4 (Goodwin et al., 2002) and intestinal MDR1 (Burk et al., 2005).

Expression of CAR1 transcript in LS180 cells. Before creating the CAR1-knockdown cell line, we determined the expression of CAR1 in LS180 cells. To our surprise, we discovered that CAR1 was not expressed in LS180 cells. CITCO is a known activator of CAR and induces the prototypical CAR target gene, CYP2B6 (Maglich et al., 2003). To confirm the absence of CAR1 in LS180 cells, we treated LS180 cells with CITCO (1 and 5 $\mu$M) for 96 h. Consistent with our observation that CAR1 is not expressed in LS180 cells, CITCO did not induce CYP2B6 transcripts. These data indicate that the PIs or rifampin induce CYP3A4 and MDR1 transcripts in LS180 cells by PXR and not CAR1 activation.

CAR1 and CAR3 activation by PIs or rifampin. Even though CAR1 is absent in LS180 cells, it is present in human hepatocytes in which the PIs and rifampin are potent inducers of CYP2B6 (Dixit et al., 2007). Therefore, we determined whether the PIs could activate CAR1. Transfection of LS180 cells with CAR1 resulted in high basal activity even in the absence of ligands (e.g., 5 $\mu$M CITCO), thus making it difficult to determine whether PIs are ligands of CAR1. A

![Fig. 4](image-url)  
* $p<0.05$; ** $p<0.001$

![Fig. 5](image-url)  
* $p<0.05$ vs. DMSO

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Materials and Methods

Expression plasmid and CYP3A4-PXRE/XREM followed by treatment with the test drugs for 48 h. Luciferase activity was measured after treatment, and data were normalized to $\beta$-galactosidase activity. PIs (10 $\mu$M) activated PXR in the following order: ritonavir ~ amprenavir > lopinavir ~ tipranavir ~ saquinavir ~ atazanavir ~ indinavir. Data are expressed relative to DMSO control treatment and presented as mean ± S.D. ($n = 3$).
one such factor is CAR1, which is particularly important in the
circumvention) for induction of these P450 transcripts. As discussed below,
inducers of CYP2B6, CYP2C9, and CYP2C19 transcripts in human
levels comparable to that of CYP3A4, only CYP3A4 transcripts were
induction of CYP3A and P-gp and because they constitutively express
We chose to use these cells as they have been used previously to study
excellent and similar (Table 1).
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similar phenomenon has been reported by other groups in immortalized hepatic cell lines such as HepG2 (Honkakoski et al., 1998). For this reason, we used a naturally occurring splice variant of CAR1, namely CAR3. This variant has low basal activity and has been successfully used to identify CAR ligands (Auerbach et al., 2003). None of the PIs activated CAR3, and CITCO activated CAR3 only 2.3- to 4-fold. The poor activation of CAR3 by CITCO led us to test whether coexpression of RXR was required for CAR3 activation and indeed it was. When RXR (pcDNA3.1-RXR) was coexpressed with CITCO (5 μM) of the CAR reporter (data not shown). This magnitude of CAR3 activation is consistent with CITCO-mediated RXR-dependent CAR3 activation observed in COS-1 cells (Auerbach et al., 2005). However, even in this refined activation assay, PIs (10 μM) did not activate CAR3 (data not shown).

Correlation in Induction of Transcripts. The correlation between the three drugs (RTV, NFV, and RIF) in induction of CYP3A4 transcripts was excellent (r = 0.8–0.86) as was that of the MDR1 transcripts (r = 0.91–0.92) (Table 1). In addition, the induction of CYP3A4 transcripts was highly correlated (r = 0.88) with that of MDR1 transcripts across all eight PIs and rifampin. Similarly, across all the drugs (nelfinavir was not included because of cytotoxicity), the correlation between PXR activation and induction of CYP3A4 (r = 0.93) or MDR1 transcripts (r = 0.93) was excellent and similar (Table 1).

Discussion

LS180 cells are an intestinal colon carcinoma cell line that expresses some characteristics of the small intestine (Tom et al., 1976). We chose to use these cells as they have been used previously to study induction of CYP3A and P-gp and because they constitutively express CYP3A, P-gp, and PXR (Pfunder et al., 2003).

Although LS180 cells expressed transcripts of many of the P450s at levels comparable to that of CYP3A4, only CYP3A4 transcripts were inducible by the various PIs and rifampin (Fig. 2). This finding is surprising as we have previously shown that RTV, NFV, and RIF are inducers of CYP2B6, CYP2C9, and CYP2C19 transcripts in human hepatocytes (Dixit et al., 2007). This observation suggests that LS180 cells lack the necessary transcriptional factors (or other cellular machinery) for induction of these P450 transcripts. As discussed below, one such factor is CAR1, which is particularly important in the
induction of CYP2B6 (Wang et al., 2004). Interestingly, tissue-specific induction of CYP3A expression and activity has been observed in vivo. Mouly et al. (2002) observed induction of in vivo hepatic CYP3A4 activity by efavirenz as measured by the erythromycin breath test without a change in intestinal CYP3A4 or P-glycoprotein expression. Similarly, chronic administration of NFV to rats induced hepatic but not intestinal CYP3a expression (Huang et al., 2001). RTV, APV, LPV, and TPV were more potent inducers of CYP3A4 and MDR1 transcripts (>50% of induction produced by RIF) than NFV, which was a modest inducer, or SQV, IDV, and ATV, which were weak inducers (Fig. 2). Among the transporters studied, the PIs and rifampin induced MDR1 transcripts (Fig. 2) but not BCRP, MRP2, or OATP1A2.

We computed the concentration-dependent induction of CYP3A4 and MDR1 transcripts by ritonavir and nelfinavir with that produced by rifampin. These two PIs were chosen as they differ in their frequency and magnitude of clinical drug interactions. Ritonavir produces more frequent and profound drug interactions than nelfinavir. In addition, in vivo, nelfinavir appears to be a less potent inducer of P450 enzymes than ritonavir. The relative induction of CYP3A4 and MDR1 transcripts by the three drugs was concentration-dependent. The EC50 values for induction of MDR1 and CYP3A4 transcripts by the inducers were remarkably consistent, whereas the Emax values differed (Fig. 3). These data support the notion that these three drugs share a common mechanism, most likely PXR, in the induction of CYP3A4 and MDR1 transcripts (see below for further discussion).

Correlation (r) between induction of CYP3A4 and MDR1 transcripts by RTV, NFV, or RIF and PXR activation and induction of CYP3A4 or MDR1 by various PIs and rifampin

<table>
<thead>
<tr>
<th>mRNA</th>
<th>RTV 3A4</th>
<th>RTV 3A4</th>
<th>mRNA/Activation</th>
<th>MDR1</th>
<th>PXR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>0.8</td>
<td>0.8</td>
<td>0.88</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>MDR1</td>
<td>0.91</td>
<td>0.91</td>
<td>0.91</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>NFV</td>
<td>0.86</td>
<td>0.83</td>
<td>0.86</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>MDR1</td>
<td>0.91</td>
<td>0.92</td>
<td>0.91</td>
<td>0.92</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6. Induction of CYP3A4 and MDR1 transcripts is reduced in PXRsi cells when compared with GFPsi cells. PXRsi (PXR levels ~20% of control cells) were treated for 96 h with moderate to strong inducers (fold induction >5) namely RIF, RTV, NFV, APV, LPV, or TPV. For all drugs, the magnitude of induction of CYP3A4 and MDR1 transcripts in PXRsi cells was 20 to 40% of that in control cells. Data are presented as mean ± S.D. (n = 4).
CYP3A transcripts into activity either because they lack the necessary machinery to do so or because the protein may be unstable in these cells. In addition, our assay for quantification of CYP3A activity did not distinguish between CYP3A4 and CYP3A5. Although CYP3A5 transcripts are expressed in LS180 cells at levels similar to those in CYP3A4 transcripts, CYP3A5 transcripts are less inducible by rifampin and negligibly induced by the PIs (data not shown), which could have dampened the overall induction in CYP3A4 activity but is unlikely to completely explain the modest induction of this activity. Also, despite washing of the cells for 4 h, residual amounts of rifampin in the cells could also have inhibited CYP3A activity. In contrast, the reduction in CYP3A activity by the majority of the PIs was probably due to inactivation of the protein as has been previously shown in vitro (Granfors et al., 2006) and in vivo (Fellay et al., 2005; Yeh et al., 2006). In addition, we cannot rule out the possibility that despite washing of the cells for 4 h, residual intracellular PIs may have inhibited CYP3A activity because these drugs are potent inhibitors of CYP3A activity (Ernest et al., 2005). Consistent with the previous data (Ernest et al., 2005), ritonavir was the most potent inhibitor of CYP3A activity whereas indinavir did not inhibit CYP3A activity. Despite our efforts to test whether P-gp activity is induced by the PIs or rifampin, we were unable to measure P-gp activity in these cells possibly because of low levels of P-gp protein expression at the cell surface. Although Perloff et al. (2003) observed induction of P-gp activity (measured by RH123 accumulation) in the LS180 cells by rifampin, they did so in cells selected (using vinblastine) for elevated expression of P-gp.

Rifampin has been shown to induce CYP3A and P-gp activity by activating PXR (Lehmann et al., 1998; Geick et al., 2001). Dussault et al. (2001) have reported that ritonavir is an excellent activator/ligand of PXR (comparable with rifampin) but not of CAR or vitamin D receptor (Dussault et al., 2001). Interestingly, none of the other PIs tested in their study (saquinavir or indinavir) activated PXR. In contrast, we observed statistically significant PXR activation with all the PIs. We suspect that the discrepancy in the two studies could be due to Dussault et al. (2001) not accounting for the poor solubility of the PIs. We used β-cyclodextrin to improve the solubility of the PIs (data not shown). The significant correlation between PXR activation and induction of MDR1 or CYP3A4 transcripts (Table 1) supports the notion that these drugs induce CYP3A4 and MDR1 transcripts in LS180 cells by PXR activation.

The above correlation is not proof. To directly test the hypothesis that PXR was involved in the induction of CYP3A4 and MDR1 transcripts in LS180 cells, we knocked-down PXR expression in these cells. In PXR knocked-down cells, the induction of CYP3A4 and MDR1 transcripts by the PIs and rifampin was significantly reduced, and this reduction was proportionate to the reduction in PXR transcripts (Fig. 6). These data unequivocally show that PIs and rifampin induce CYP3A4 and MDR1 transcripts in LS180 cells by PXR activation. Because the CAR is also important in inducing CYP3A4 and MDR1 (Goodwin et al., 2002; Burk et al., 2005; Cerveny et al., 2007), we investigated whether CAR1 is expressed in LS180 cells. Surprisingly, we found that CAR1 transcripts were not expressed in LS180 cells. These data further support our conclusion that induction of CYP3A4 and MDR1 transcripts by the PIs and rifampin in LS180 cells is PXR- and not CAR1-mediated.

Although CAR1 is not expressed in LS180 cells, it is expressed in the human liver where it may play a role in the in vivo induction of CYP3A4 and MDR1 transcripts by the PIs. It is difficult to study CAR1 activation in hepatic-derived immortalized cells, as CAR1 spontaneously translocates to the nucleus in the absence of ligand (Honkakoski et al., 1998), resulting in high constitutive CAR activity. The most frequently used cell system to study CAR1 activation is primary human hepatocytes. Their limited availability, variability, and lack of suitability for high-throughput assays prompted us to test whether LS180 cells could potentially be used for CAR1 activation. Unfortunately, results from our experiments suggest that CAR1 translocates to the nucleus in the absence of ligand in LS180 cells similar to what occurs in HepG2 cells, and, hence, LS180 cells cannot be used for CAR1 activation studies. To overcome this problem, measurement of activation of CAR3, a splice variant of CAR1, has been proposed. CAR3 has low basal activity (Auerbach et al., 2005) and does not spontaneously translocate to the nucleus. Therefore, we determined the ability of the PIs to activate CAR3 in LS180 cells. None of the PIs tested was able to activate CAR3, indicating negligible involvement of CAR in PXR-mediated induction of CYP3A4 and MDR1. These data further support our conclusion that PIs and rifampin induce CYP3A4 and MDR1 transcripts by PXR- and not CAR-mediated activation.

Because the PIs inactivate CYP3A enzymes, both in vitro and in vivo, their ability to induce CYP3A4 transcripts will not translate into induction of CYP3A activity. Therefore, such induction will not correlate with in vivo CYP3A activity after chronic administration of these drugs. However, does the ability of PIs to induce MDR1 transcripts in LS180 cells correlate with their ability to induce P-gp activity in vivo? In humans, single dose administration of ritonavir or lopinavir/ritonavir increased the plasma AUC (2.2- to 4-fold) and Cmax of the P-gp substrate, fexofenadine. However, a moderate induction of P-gp activity was suspected after 12 days of lopinavir/ritonavir 400/100 mg twice daily, although a net inhibitory effect on P-gp activity was still maintained (van Heeswijk et al., 2006). Additionally, when digoxin (0.4 mg), a P-gp substrate, was administered orally before and after ritonavir treatment (200 mg twice daily for 14 days), digoxin AUC0–72 was increased by 22% and oral digoxin clearance was reduced by approximately 30% (Penzak et al., 2004). In another study, 300 mg of ritonavir twice daily for 11 days resulted in increased AUC0–∞ (86%) and volume of distribution (77%) of digoxin (0.5 mg) administered i.v. on day 3 of ritonavir administration. Furthermore, the nonrenal (48%) and renal clearance (35%) of digoxin was reduced (Ding et al., 2004). Collectively, these data suggest that with chronic administration of the drug, ritonavir is a net inhibitor, not inducer, of intestinal P-gp.

In conclusion, this is the first comprehensive study of PXR-mediated induction of CYP4A4 and MDR1 transcripts in LS180 cells. We show for the first time that neflinavir and several other PIs such as amprenavir, tipranavir, lopinavir, atazanavir, and saquinavir induce CYP3A4 and MDR1 transcripts by PXR (but not CAR) activation. In addition, our results indicate that LS180 cells do not express CAR1 and therefore can serve as an excellent, inexpensive cell line to screen for PXR-mediated induction of CYP3A4 and MDR1 gene products.

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References


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